Enzymes for Lignin Depolymerization

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Lignin, a rigid polymer composed of phenolic subunits with high molecular weight and complex structure, ranks behind only cellulose in the contribution to the biomass of plants. Therefore, lignin can be used as a new environmentally friendly resource for the industrial production of a variety of polymers, dyes and adhesives. Since laccase was found to be able to degrade lignin, increasing attention had been paid to the valorization of lignin. The enzymes involved in lignin depolymerization are mainly divided into four categories: laccase, lignin peroxidase (LiP), manganese peroxidase (MnP) and versatile peroxidase (VP).

Keywords: Lignin ; Laccase ; Enzymes

1. Lignin

Coniferyl (G), sinapyl (S) and *p*-coumaryl (H) alcohols are the three categories of monolignols in lignin, which are divided into guaiacyl (G-type), guaiacyl-syringyl (GS-type) and grassy or guaiacyl-syringylp-coumaric (GSH-type). However, it needs to be noted that, in actual examples, other aromatic units are occasionally included in lignin, which is a common phenomenon. For example, Stewart et al. described the structure of the lignin polymers from poplar. The lignin from wild-type poplar contains *p*-hydroxybenzoate [1]. C-lignin, found in plants such as *Vanilla planifolia* and *Cleome hassleriana*, is wholly derived from caffeyl alcohol [2]. For sure, the GSH-type proportion and linkages proportion in these practical examples also varied from each other.

As a concept different from lignins in vivo, technical lignins (e.g. kraft lignin, soda lignin, lignosulfonates and organosolv lignin) are isolated lignins, most of which are byproducts from the paper and pulp industry [3][4]. Kraft lignin, which is recovered from the black liquor, is generated from the paper and pulp industry as a by-product that is generated by treatment with Na_2S and NaOH [5]. However, the extraction of lignin from black liquor limits the commercial development of lignin until the emergence of the LignoBoost and LignoForce methods, which could also be applied for lignosulfonate and soda lignin. An economical benefit could be achieved as the extracted lignin has a high proportion of phenolic hydroxy groups [6].

Lignosulfonate is obtained as a by-product in the spent liquor of the pulping process by treating plant fiber as raw materials with an aqueous solution of sulfur dioxide and a calcium, magnesium, ammonium or sodium base [7]. The kraft lignins could also be used as raw metarials for lignosulfonate production. In the research provided by Ruwoldt [8] and Kienberger et al. [9], the lignin extraction processes and their dominant products, including commercialized lignosulfonates, were well-summarized. Membrane processes for the isolation and purification of lignosulfonates are also commercially available. In the meanwhile, fractionation of the lignosulfonates could obtain products with high quality. Recently, Demuner et al. elucidated two different processes for lignosulfonate production from eucalypt kraft lignin by sulfomethylation and acid sulfonation [10]. Varied pH levels can be applied in this process, for example, lignosulfonate production through neutral sulphite pulping was investigated [11]. It has the higher sulfur content and the lower price at the market when compared with kraft lignin [12].

Soda lignin is obtained from the soda or soda-anthraquinone pulping processes, in which 13–16% sodium hydroxide (pH 11–13) is used to extract lignin [13]. The chemical composition of soda lignin is closer to the natural lignin when compared with kraft lignin and lignosulphonate [14]. The major advantage of soda lignin is that it is obtained from wheat straw, an unwanted agricultural waste derived from the cropping of wheat.

Organosolv lignin is attained from the organosolv process, in which organic solvents are applied. Organosolv lignins, which are hydrophobic and water-insoluble, have several important characteristics: low molecular weight, high quality and high purity [15]. Nonetheless, the application of organic solvents may face the problem of environmental pollution [10]. However, organosolv lignin is sulfur-free, as is soda lignin, thus relieving the concern of environmental pollution from

another perspective. In a research given by Wang et al., they well-summarized the chemical extraction methods for technical lignin [16].

Common derivatives such as vanillin, lipid and PHA could be produced via the lignin degradation process [17]. Multiple high value-added small molecules have been linked with lignin. Lignin, with a great amount of phenols, is considered to be a good candidate for producing phenolic resins. At present, products from lignin depend on sources besides the operating conditions [18]. Producing lignin-based resin with a high substitution rate of lignin to phenols is still a challenge. Recently, lignin with more S units was converted to desirable phenolic resins with a suitable viscosity and high substitution rate using chemical means with the beginning of methylolation [19]. Like phenolic resins, bio-oil generated by lignin can also broaden the application of lignin and thereby meet the new concept of carbon neutrality. Lignin can be transited to bio-oil via thermochemical methods (pyrolysis, gasification and liquefaction) [20]. However, these processes are usually energy-consuming and labor-intensive [21]. Problems like high energy demand and environment pollution encourage people to explore new ways for lignin degradation and valorization. The biodegradation of lignin could be an ideal choice. By exploiting enzymes by means such as cell-free systems, and taking advantage of the pathway of microorganisms to produce high value-added compounds, the biodegradation and valorization of lignin has a broad prospect.

2. Laccase (EC 1.10.3.2)

As a typical lignin oxidase, increasing attention has been paid to lignin depolymerization by laccase (EC 1.10.3.2). Laccase, a type of multi-copper oxidase (MCO), generally has four copper atoms (one type-1 copper, one type-2 copper and two type-3 coppers) in the catalytic site and oxidizes a variety of substrates by performing the single-electron transfer reaction four times. A O_2 molecule is reduced to two molecules of H_2O through the electron transfer pathway in laccase [22][23]. The laccase-like activity could be detected by applying agar plates containing standard laccase substrates guaiacol or syringaldazine and then monitoring the dark colors coming from the oxidation of these substrates [24]. Chen et al. analyzed the binding mechanism of laccase from *Trametes versicolor* (formerly *Coriolus versicolor* or *Polyporus versicolor*) with lignin model compounds, and it turned out that hydrophobic contacts were necessary between laccase and the model compounds, but H-bonds were alternative [25]. In the reaction between laccases and lignins, the phenoxy radicals are first to be generated. The enzyme kinetics of lignin radical formation can be achieved by electron paramagnetic resonance (EPR) [26]. The formation of radicals could result in (1) depolymerization of the lignin [27], (2) polymerization of the lignin [28] or (3) the change of the functional group [29]. These potential outcomes could be effected by factors such as reaction conditions or whether the Laccase-Mediator System is involved in the reaction system.

The depolymerization of lignin requiring mediators is a common opinion [27][30]. Due to the relatively low redox potential of laccase for aromatic compounds, the Laccase–Mediator System (LMS) has been widely studied in order to expand the applications of laccases [31][32][33][34][35]. The mediators can be divided into three categories: natural mediators, synthetic mediators and other mediators. Some effective mediators have been widely used in industries, such as 1-hydroxybenzotriazole (HBT), methyl syringate and 2,2′-azino-bis(3-ethylbenzo thiazoline-6-sulfonic acid) (ABTS). In the LMS, the mediator is firstly oxidized by laccase and then undergoes a redox reaction with the substrate. This mechanism also implies that the laccase is a type of environment-friendly biocatalyst due to its lower requirements for factors such as heat for pyrolysis. Although mediators can tremendously accelerate the process of substrate oxidation by laccase [36], it also has significant influences on the stability of laccase. The free radicals generated from the oxidized mediator could attack laccase and then lead to its inactivation. To avoid the radical attack and improve the stability of laccase in LMS, it could be a promising strategy to find mediators that generate free radicals with lower activities.

According to the evolutionary tree created, laccases from different organisms are highly homologous to each other, which suggests that their protein sequences are highly conserved. The identification of the specific amino acids responsible for the increase of the oxidation potential of laccase will contribute to the industrial application of laccase. Laccases from fungi, plants, insects and bacteria have been characterized. Laccases from white-rot fungi *Pycnoporus cinnabarinus* and *T. versicolor* have attracted wide attention since the last century [37]. *P. cinnabarinus* produces laccase in one isoform, and neither LiP nor MnP are detected. Thus by comparison between wild-type strains and laccase-less mutants, laccases from *P. cinnabarinus* are proven to be efficient for lignin degradation [38]. Kawai et al. reported that laccase from *T. versicolor* can catalyze various reactions of nonphenolic-O-4 lignin in the presence of HBT, such as β-ether cleavage, Cα-Cβ cleavage, aromatic ring cleavage and Cα-oxidation [39]. Compared with fungal laccases, laccases from bacteria have a higher thermostability and wider effective pH range [40][41][42]. Moreover, they are efficiently expressed in heterologous hosts such as *E. coli* with relatively high yields. All of these advantages render bacterial laccases great potential in energy and environmental biotechnology. Fungal laccases and bacterial laccases also tend to cause different effects on lignin; a study showed that fungi mineralized kraft lignin, while bacteria partially degraded and modified lignin [43]. In addition, due to their low substrate specificity, laccases have also been applied in many fields such as bioremediation, wood and paper

industries [44][45]. The application of laccase is also reflected in the potential of small lignin production. With *Trametes villosa* laccase combined with HBT, the yield of glucose and ethanol were increased from lignocellulosic by Gutiérrez et al. [27] Through catalyzation of the above-mentioned enzymes, lignin enters the degradative pathways. To better understand the degradative pathways for lignin-derived aromatic compounds, it is necessary to summarize the products and effect of the enzymes in lignin depolymerization. The biodegradation of alkaline lignin by *Bacillus ligniniphilus* L1 resulted in the production of phenylacetic acid, 4-hydroxy-benzoicacid and vanillic acid [46]. Based on the whole-genome sequencing (with no LiP and MnP) and the decolorization experiment, laccase is expected to be responsible for the depolymerization of alkaline lignin in *B. ligniniphilus* L1.

3. Lignin Peroxidase (EC 1.11.1.14)

Lignin peroxidase (LiP) (EC 1.11.1.14), which belongs to the peroxidase-catalase superfamily, was firstly discovered in white-rot fungus *Phanerochaete chrysosporium* during the 1980s [47][48]. LiP is a heme peroxidase, which contains a protoporphyrin IX (heme) as the prosthetic group. Heme iron is responsible for the electron transfer and the high redox potential of LiP. Hydrogen peroxide (H_2O_2) functions as the electron acceptor in the catalytic site of LiP and drives the oxidation step. Two steps are required for the reduction of LiP. Compared with laccase, LiP has a higher reduction potential and requires no mediators [49]. However, previous studies found that the addition of veratryl alcohol (3,4-dimethoxybenzyl alcohol; VA) can enhance the activity of LiP by transforming the electron between LiP and the substrates, thus promoting the rate of lignin depolymerization [50]. LiP exhibits oxidative activity on a variety of compounds, such as phenolic compounds, amines, aromatic ethers and polycyclic aromatics [51][52]. Only two single-electron transfers are required for substrate oxidation. Though H_2O_2 plays an important role in the typical peroxidase catalytic cycle, excess H_2O_2 inhibits the activity of LiP [53]. To exploit the lignin degradation ability of LiP, explorations from different strategies were carried out. Majeke et al. reported the synergistic application of quinone reductase (QR) and LiP; this combination decreased the molecular weight (MW) of four different lignins [54] and showed the potential to benefit from the cooperation of different enzymes. On the other hand, considering LiP could be utilized in cell-free systems, the production of LiP can be improved. The effort for the large-scale production of LiP could boost the application of LiP for reasons such as cost reduction. Liu et al. achieved a maximum LiP activity of 1645 mU/L via multipulse-fed batch production [55]. Based on these paradigms, LiP will have larger application prospects.

4. Manganese Peroxidase (EC 1.11.1.13)

Manganese peroxidase (MnP) (EC 1.11.1.13) was also discovered in *P. chrysosporium* [56]. MnP could be secreted by ligninolytic fungi into their microenvironment and, thus, be an ideal choice for cell-free systems. Similar to LiP, MnP is also a heme-containing enzyme, with disulfide bonds and Ca^{2+} maintaining the conformation of the active site [57]. When H_2O_2 binds to the ferric enzyme in a resting state, the reactions will be initiated by forming an iron–peroxide complex. MnP compound III, an inactive oxidation state, could be formed under excess H_2O_2 condition [58]. Therefore, first, Mn^{2+} is oxidized to Mn^{3+}; then, Mn^{3+} acts as the redox mediator to attack phenolic lignin structures and results in the formation of instable free radicals, causing lignin degradation. One remarkable difference in the catalytic mechanism between LiP and MnP is that MnP, but not LiP, requires the conversion between Mn^{2+} and Mn^{3+} as an intermediate of the reaction. In the absence of mediators, one molecule of water will be produced in LiP's catalytic cycle, but the number of water is two for MnP's catalytic cycle [59]. By the treatment of milled wood with the MnP–, Mn^{3+}– or Fe^{2+}–linoleic acid system, MnP exhibited the most intense reactions, including transformation in the lignin structure and water-soluble low molar mass aromatic fragments releasing [60]. Base on the increase of the carboxyl content, the degradation of lignin was carried out via β-O-4 and Cα–Cβ cleavage and side-chain oxidation. In addition, besides the degradation of lignins, attention has been paid to the versatility of MnP. For example, MnP from *P. chrysosporium* could improve the efficiecy of cellulosic decomposing [61]. A manganese peroxidase-producing yeast consortium (MnP-YC4) has been developed to withstand lignin degradation inhibitors while degrading and detoxifying azo dye [62].

5. Versatile Peroxidase (EC 1.11.1.16)

Versatile peroxidase (VP) (EC 1.11.1.16), also belonging to the peroxidase-catalase superfamily, was firstly found in *Pleurotus eryngji*. Studies have revealed that VP is a "hybrid peroxidase", because its gene sequence contains the partial sequences of LiP and MnP. It integrates the substrate specificity and catalytic properties of LiP and MnP, respectively [63]. Therefore, VP can oxidize phenolic, nonphenolic and lignin derivatives without manganese ion or any mediator because of the high redox potential and broader substrate spectrum.

The high redox potential of VP results in a wide substrate specificity. For eample, VP can oxidize the LiP substrate veratryl alcohol; five molecules of water will be produced in the presence of veratryl alcohol (VA) [36][64]. In a previous research, VP showed oxidizing activity on hydroquinone in the absence of exogenous H_2O_2 but in the presence of Mn (Ⅱ) [65]. When oxidizing phenolic compounds, VP uses a similar mechanism to MnP.

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